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(54) Title: CONTRACEPTIVE VACCINE BASED ON CLONED ZONA PELLUCIDA GENE

(57) Abstract

The present invention relates to contraceptive vaccines based on cloned zona pellucida genes and the strategy of alloimmunization with zona pellucida polypeptides. In particular, the present invention relates to a contraceptive vaccine for use in a mammalian female comprising a polypeptide which displays at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This epitope is from a zona pellucida protein of the species in which the said vaccine is used. This invention relates, more particularly, to such vaccines wherein the zona pellucida protein is either the ZP3 or the ZP2 or the ZP1 protein of the mouse or homologues of these proteins in some other mammalian species. Further, this invention comprehends vaccines comprising a synthetic peptide that displays an epitope for such an antibody that inhibits fertilization. In addition, this invention relates to cloned DNA segments variously encoding the mouse ZP3 or ZP2 proteins or the human ZP3 protein.

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CONTRACEPTIVE VACCINE BASED ON CLONED ZONA PELLUCIDA GENE

FIELD OF THE INVENTION

The present invention relates to contraceptive vaccines based on cloned zona pellucida genes and the strategy of alloimmunization with zona pellucida polypeptides. In particular, the present invention relates to a contraceptive vaccine for use in a mammalian female comprising a polypeptide which displays at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This epitope is from a zona pellucida protein of the species in which the said vaccine is used. This invention relates, more particularly, to such vaccines wherein the zona pellucida protein is either the ZP3 or the ZP2 or the ZP1 protein or the mouse or homologues of these proteins in some other mammalian species. Further, this invention comprehends vaccines comprising a synthetic peptide that displays an epitope for such an antibody that inhibits fertilization. In addition, this invention relates to cloned DNA segments variously encoding the mouse ZP3 or ZP2 proteins or the human ZP3 protein.

BACKGROUND OF THE INVENTION

There is currently much interest in the development of a safe and effective contraceptive vaccine for control of diverse mammalian populations. Contraceptive vaccines would be useful under certain circumstances where relatively long-term but not permanent contraception is desired without the need for frequent intervention, for example, in pets including cats and dogs, in agriculturally important livestock such as cattle and pigs, and in human beings. A contraceptive

vaccine preferably should have an effect which is long-lasting and highly specific. Further, to minimize possibilities for birth defects in the event of failed contraception, the antigen which is selected as the immunogen should produce contraceptive antibodies that inhibit fertilization of the egg by a sperm rather than by an abortifacient mechanism involving disruption of early development. In addition, the vaccine preferably should induce an immunological response that is sufficient to be effective for contraception without eliciting a cytotoxic response that might result in abnormal reproductive function.

The mammalian zona pellucida, which surrounds growing oocytes and ovulated eggs, has been recognized as a potential immunogen for a contraceptive vaccine (C.J. Henderson, et al., 1988, J. Reprod. Fert. **83**, 325-343; B.S. Dunbar, 1983, Mechanisms and Control of Animal Fertilization, J.F. Hartmann, ed., pp. 140-175, Academic Press, New York; A.T. Sacco, 1987, Am. J. Reprod. Immunol. Microbiol. **15**, 122). At birth the mouse ovary contains 10,000-15,000 oocytes in the prophase of the first meiotic division. As cohorts (10-15) of these oocytes enter into a two week growth phase, they synthesize and secrete zona proteins to form the extra-cellular zona pellucida which ultimately reaches a thickness of 7 μm in the fully grown oocyte. The zona is unique to the ovary, being highly antigenic and accessible to circulating antibody during the two week intra-ovarian oocyte growth phase prior to meiotic maturation and ovulation.

Passive immunization of mice or hamsters with anti-zona sera has been shown to produce reversible

contraception without obvious side effects. For example, U.S. Patent 3,992,520 to Gwatkin discloses, inter alia, an anti-serum composition for short-term control of fertility comprising antibody obtained by immunizing an animal with water solubilized zona pellucida of a distinct donor species. This method requires isolation of large amounts of a relatively scarce natural antigen, however. Further, long-term administration of antibodies from a foreign (i.e., "heterologous") species leads to induction of reactive antibodies that will inhibit the contraceptive action of the contraceptive antibodies. Further, administration of serum or products isolated from serum carries inherent risks of transmission of blood-born diseases.

Structural information about the zona pellucida has been available for some years. The mouse zona, for instance, is composed of three sulfated glycoproteins, designated ZP1, ZP2 and ZP3, (J.D. Bleil et al., 1980, Dev. Biol. **76**, 185; S. Shimizu et al., 1983, J. Biol. Chem. **258**, 5858) which play important roles in fertilization and early development and have average M_rs of 200,000, 140,000, and 85,000, respectively. ZP2 and ZP3 appear to be complexed into long filaments which are crosslinked by ZP1 in the zona matrix providing structural integrity to the zona pellucida. Sperm initially bind to ZP3 via O-linked oligosaccharide chains and continued binding involves ZP2 as a secondary sperm receptor. Subsequently, ZP3 induces lysis of the sperm's acrosome which releases enzymes (such as glycosidases and proteases) which are thought to be important for the penetration of the zona pellucida by sperm. Following fertilization, both ZP2 and ZP3 are biochemically

modified to prevent additional sperm binding and thereby to facilitate the post-fertilization block to polyspermy.

The zona pellucida in other mammals besides the mouse is known to comprise several distinct glycoprotein components with apparent sizes and, hence naming terminologies, that do not necessarily correspond directly to the mouse ZP1, ZP2 and ZP3. In some cases, an additional protein has been observed in other species such as the pig (designated, e.g., ZP4); whether this represents a degradation product of the equivalent of ZP1, ZP2 or ZP3 has not been determined. Recently, however, the porcine ZP3 glycoprotein has been purified to apparent electrophoretic homogeneity and further analyzed (E. C. Yurewicz et al., 1987, J. Biol. Chem., **262**, 564-571). Collectively, the data were interpreted to indicate that the 55,000 Da ZP3 antigen of porcine oocyte zona pellucida is in fact comprised of overlapping families of charge isomers corresponding to two structurally and immunologically distinct lactosaminoglycan- containing glycoproteins.

In light of the identification of the distinct murine zona pellucida polypeptides, ZP1, ZP2 and ZP3, further experiments on passive immunization with contraceptive antibodies have been conducted. Specifically, rat anti-mouse ZP2 and anti-mouse ZP3 monoclonal antibodies were injected into female mice and were found to bind specifically to the zonae surrounding growing, intra-ovarian oocytes. After ovulation, the binding of the antibody to the zona persisted; and the presence of these antibodies precluded fertilization by preventing sperm from penetration of the zona pellucida. This contraceptive effect was long-term, lasting

approximately 15 mouse estrus cycles, but was eventually reversible. There was no evidence of any adverse effect on the development of fertilized embryos to term and no evidence of abnormal ovarian histology or function. However, the antibody binding sites (i.e., "epitopes") recognized on mouse ZP2 and ZP3 by five different rat anti-mouse monoclonal antibodies that were tested are not present on other mammalian zonae pellucidae (7,8). This species specificity limits the usefulness of these particular antibodies as contraceptive agents essentially to murine species. In addition, even if analogous murine anti-ZP2 or anti-ZP3 antibodies that inhibit fertilization could be identified for ZP2 or ZP3 of non-murine species, there are inherent side-effects from the repeated administration of heterologous antibodies, as noted above.

There have been several studies on active immunization using preparations of isolated zona pellucidae to immunize rodents (C.J. Henderson, et al., 1988, J. Reprod. Fert. **83**, 325; R. B. L. Gwatkin, et al., 1977, Fert. Steril. **28**, 871). Further, the U.S. Patent to Gwatkin cited above (U.S. 3,992,520) also discloses a vaccine for the immunological control of fertility in female mammals that consists of an aqueous solution of water solubilized zona pellucida prepared by heating mammalian zona pellucida at 65-100° C in an aqueous medium. One example therein describes a bovine antigen preparation intended for use in humans. In addition, Japanese Patent 63,150,299 discloses a pig zona pellucida antigen for use as contraceptive vaccine for pigs or humans that is characterized as a glycoprotein of 20 to 30 kDa in molecular weight which can be extracted

from soluble pig zona pellucida with 8.5 M urea and 2% 2-mercaptoethanol.

Despite positive results under experimental conditions, these methods of preparing a vaccine from natural zona pellucida materials are clearly difficult if not outright impractical for commercial use, particularly in the human case, due to limited sources of antigen and to difficulties in quality control of such poorly defined vaccines. Further, widespread ovarian histopathology and dysfunction were reported in rabbits, dogs and primates after active immunization with zonae pellucidae or extracted antigens (see, for example, R.B.L. Gwatkin, et al. , 1980, Gamete Res. **1**, 19; A.T. Sacco, 1977, Am. J. Reprod. Immunol. Microbiol. **15**, 122). Several studies have suggested that both the dose and the purity of the immunogen contributed to these abnormalities, two properties that are particularly difficult to control in such relatively crude antigen preparations.

The effect of the genetic origin of the zona pellucida antigen on its ability to immunize a given species against conception has been examined in several studies. For instance, the efficacies of contraceptive immunizations with pig and rabbit zonae pellucidae on fertility in rabbits was compared. This comparison of results with "alloimmunization" (literally "self-immunization", using antigen from the same species, i.e., an "alloantigen") with those of "heteroimmunization" (using antigen from another species, i.e., an "heterologous" antigen) suggested (D. M. Wood et al., 1981, Biol. Reprod. **25**, 439-450) that heteroimmunization of rabbits with porcine zonae is more effective in reducing fertility than alloimmunization

with rabbit zonae. More recent work using immunoaffinity purified antibodies to zona pellucida to compare immune responses in alloimmunization of male and female rabbits has continued to support the greater effectiveness for contraception of heteroimmunization with zona pellucida antigens. (S. M. Skinner, et al., 1987, J. Reproductive Immunology **12**, 81-92).

Another general approach toward providing a vaccine related to any antigen involves the use of a particular type of antibody, called an "anti-idiotypic" antibody, as an immunogen to actively immunize an animal. Anti-idiotypic antibodies are antibodies directed to the antigen binding site of another antibody; accordingly, the antigen binding site of the anti-idiotypic antibody mimics or represents an image of the site on the antigen that is bound by the other antibody. U. S. Patent 4,795,634 to Grimes et al. (equivalent of WO 87/05,516) discloses a vaccine that comprises anti-idiotypic antibodies to anti-zona pellucida antibodies to express images of zona pellucida antigens. This vaccines suffers from drawbacks including the fact that anti-idiotypic antibodies are generally difficult and expensive to prepare in amounts and purity satisfactory for vaccine usage, particularly in human applications. Further, heteroimmunization with antigens comprising antibodies from another species may induce predominantly antibodies to sites on the antibody other than the desired target, the antigen binding site. In other words, the desired antigen binding site may not constitute an "immunodominant" antigenic site (or "determinant") for the vaccine antibody protein in a species different from that which produced the vaccine protein (see below for a

discussion on the basis of immunodominance). Another technique for producing vaccines that is known generally in the art is the use of specific isolated polypeptides as antigens, or of peptides representing portions of such polypeptides, in place of crude antigen preparations comprising aqueous extracts of target tissues. Accordingly, European Patent EP-0117934 to Stevens discloses a modified antigen for use in fertility control comprising an unspecified antigen from the zona pellucida, or a peptide having a sequence corresponding to at least part of the sequence of such a zona pellucida antigen, which antigen or peptide has been chemically modified outside the body of the animal. The modified antigen has a greater capacity to induce antibodies than the unmodified antigen from which it is derived. According to the specification and claims, such modification includes coupling the antigen or peptide through a maleimido linkage to a suitable "carrier" protein that is biologically foreign to the animal to be vaccinated and of size sufficient to elicit antibody response. Neither this application nor any related applications as yet published teaches specific zona pellucida polypeptides or peptides that are suitable for use as contraceptive vaccines.

In light of the complexities, difficulties and uncertainties of all the contraceptive vaccines described above, there is yet a need for a simpler, safer, cheaper, more defined and effective contraceptive vaccine. Toward this end, the present inventor and associates have recently constructed a mouse ovarian cDNA expression library and isolated two overlapping ZP3 cDNA clones (M. J. Ringuette et al., 1986, Proc. Natl. Acad. Sci. USA **83**,

4341), one of which expresses a fusion protein recognized by an anti-ZP3 monoclonal antibody (I. J. East et al., 1985, Dev. Biol. **109**, 268).

The identity of these clones was confirmed by a comparison of the amino acid sequence encoded by a 60 nucleotide stretch of their nucleic acid sequence with the terminal amino acid sequence (20 amino acids) of a large internal fragment isolated from the ZP3 protein (Ringuette et al., 1986, supra). This fragment was isolated from purified ZP3, following digestion with a protease, by affinity chromatography using an anti-ZP3 monoclonal antibody. Therefore, it was clear that this fragment was capable of expressing an epitope for a contraceptive antibody; however, the location of that epitope within scores of amino acid residues was not known. More importantly, the ability of this proteolytic cleavage fragment to serve as an immunogen in a vaccine was not known, nor was there any practical means for preparing sufficient material from natural sources to test that cleavage fragment further.

A first attempt to utilize the cloned mouse ZP3 cDNA described above to produce a vaccine was unsuccessful (S. M. Chamow and J. Dean, 1987, abstract of presentation to the American Society of Biological Chemists). This effort involved testing of the recombinant ZP3-β-galactosidase fusion protein, which contained most of the ZP3 amino acids as well as a larger portion of β-galactosidase and was generated according to well known methods in genetic engineering that have successfully produced other antigens with native immunoreactivity. Immunization with this particular fusion, however, failed to induce detectable antibodies

that would react with native ZP3; reactivity was detected only after reduction of disulfide bonds and denaturation.

The basis of this failure to induce anti-ZP3 contraceptive antibodies, despite that fact that the cDNA clearly encoded a proteolytic cleavage fragment that reacted with such an antibody, is not entirely clear. It may be that, under the conditions of immunization, the portion of the fusion protein that encoded the contraceptive antibody epitope did not assume the proper conformation to react with such antibodies. In other words, although the fusion protein surely encoded the amino acids that formed the epitope in the native ZP3 protein, it may be that those amino acids did not exhibit (i.e., did not "display") that epitope in this instance. It is also possible that epitopes for other antibodies, which were located on the β-galactosidase moiety of the fusion, may have been immunodominant over the contraceptive antibody epitopes and thus prevented a detectable contraceptive antibody response (see discussion of immunodominance below). Finally, a combination of these effects and others may have united to prevent the desired contraceptive antibody response to the fusion product of the recombinant DNA which expressed most of the ZP3 polypeptide. These results clearly illustrate the unpredictability of the immunogenicity of a polypeptide under any given set of conditions, no matter how efficacious they may be for other antigens, and the need for experimental determination of the necessary physical form of the amino acids that encode an epitope (e.g., polypeptide size and nature of attached amino acid sequences) to display that epitope and, further, to induce antibodies to it.

Accordingly, it is an object of the present invention to find an efficacious way to use contraceptive antibodies and cloned genes encoding zona pellucida proteins to develop contraceptive vaccines for use in a mammalian female. More particularly, it is an object of this invention to provide such vaccines comprising polypeptides that include defined amino acid sequences that are selected for their ability to display epitopes for contraceptive antibodies.

Additional immunological analyses of the individual ZP polypeptide components have been carried out. For example, specific monoclonal and polyclonal antibodies have been employed to define distinct antigens of the porcine zonae pellucidae, leading to the suggestion that there are both unique and shared antigenic determinants present in the individual components of the zona pellucida, but that the immunodominant determinants appear to be unique to each glycoprotein (T. M. Timmons, et al., 1987, Biology of Reproduction **36**, 1275-1287).

Finally, there has been a report of an effort to molecularly clone cDNAs encoding specific antigenic sites from rabbit ZP proteins using antibodies that recognize determinants found on ZP antigens of several species (P. Cheung et al., 1987, abstract of a presentation at the twenty-seventh annual meeting of the American Society for Cell Biology, St. Louis, Missouri, November 16-20, J. Cell Biol. **105**, no. 4 part 2, 334A). This abstract reported in part that:

> "These studies demonstrated that cross-species affinity purification of antibodies is an effective method for isolating cDNA clones expressing antigens

which are shared among different mammalian species." However, no specific nucleotide or amino acid sequences were disclosed in this abstract, nor was the contraceptive potential of the antibodies discussed; indeed, there was no mention of any contraceptive vaccine. In a speculative exposition on the use of recombinant DNA and synthetic peptide technologies for development of a human contraceptive vaccine from porcine zona pellucida antigens (C.J. Henderson, et al., 1988, J. Reprod. Fert. **83**, 325), which was entitled "The future ...", the identification of amino acid sequences displaying epitopes for contraceptive vaccines on a particular porcine polypeptide is anticipated, although absolutely no sequences of the polypeptide are disclosed. Nevertheless, this reference goes on to hypothesize that known vaccine technologies, including synthetic peptides and vaccinia virus expression vectors, will provide successful human vaccines based on this particular porcine polypeptide that is known to be immunologically related to human zona pellucida antigens. Furthermore, while asserting that monoclonal antibodies to this polypeptide that exert a contraceptive effect "will be extremely important in defining the epitopes with contraceptive potential ...", this report also notes that, despite obtaining monoclonal antibodies reactive with this polypeptide, the authors "have failed to generate a monoclonal antibody with contraceptive effect; this is in accord with other published reports ...."

Although a complete exposition of the current theoretical basis of immunogenicity and antigenicity of polypeptides is beyond the scope of the present disclosure, a brief discussion of selected principles and

terms of this active art will facilitate further understanding of the instant invention. [In this application, absent an express statement to the contrary, each use of the term "polypeptide" encompasses any polymer comprising two or more amino acids coupled by peptide linkages (i.e., dipeptides, oligopeptides, peptides, polypeptides) as well as proteins consisting of multiple polypeptide subunits.] Accordingly, it should be noted first that the necessary and sufficient properties of a polypeptide for inducing antibodies cannot be predicted for any given set of conditions (e.g., for a particular species, or for presentation in a certain form). Nevertheless, much more has been learned about this subject in the past decade than is reflected in any of the art cited so far herein, and it is a further object of the present invention to exploit aspects of this knowledge for design of advantageous contraceptive vaccines.

In particular, comprehension of the present invention will be aided by the now widely held view that the nature and level of the immune response to a polypeptide depends on its interactions with at least two distinct classes of immune system cells, namely B-cells and T-cells. In simple terms, the role of B-cells in immunity may be thought of as recognition of the specific sites on macromolecules to which antibodies are produced and subsequent production of those antibodies. These B-cell recognition sites, which provide the main basis for immune recognition of nonself molecules and are also called B-cell epitopes, are of a size corresponding to about that of the antigen binding site on an antibody, typically of a diameter equivalent to the length of a

peptide containing about four to six amino acids.

[It may be noted here that there exists a formal distinction between the epitope for a B-cell and that of its related antibody. In other words, due to complex biological mechanisms that intervene between the recognition by a B-cell of a given site on an antigen and the consequent production of antibodies to that site, it is possible that the ultimate antibody recognition site may not be precisely identical to the initially recognized B-cell epitope. However, for the present purposes, B-cell epitope may be considered to be essentially the same structure as the binding site for the corresponding antibody.]

The functions of T-cells, on the other hand, relate in large measure to helping to activate antibody production by B-cells upon initial exposure to an antigen, as well as to enhancing their antibody response upon subsequent reexposures (i.e., to "immune memory" or the "amnestic" response). To play their roles in immunity, T-cells must also recognize specific sites on an antigen to which antibodies are produced, and such T-cell epitopes are about the same size as B-cell epitopes.

B-cell and T-cell epitopes on any given polypeptide, however, need not comprise the same amino acid residues. In fact, it will be appreciated by those of ordinary knowledge in the current art of peptide immunology at the molecular level, that even in a peptide consisting of only half a dozen amino acids, there may coexist several different B-cell epitopes (comprising, for instance, from two to four atoms that contact complementary structures on the antibody) and one or more

distinct T-cell epitopes which may or may not include atoms of amino acids also included in a B-cell epitope.

It is also well known that the vast majority of small peptides (containing six to twenty amino acids, for instance) that have been tested for induction of antibodies are considerably less potent immunogens than the larger proteins from which they have been derived, despite ample ability of the peptides to bind to antibodies directed against those larger proteins. Certain chemical modifications of a peptide, particularly coupling of the peptide to a larger proteinaceous "carrier", generally enhances the immune response to a small peptide.

Although the role of such a carrier still may not be fully understood in all respects, it has been clearly established in particular, that there is no specific minimum size requirement for peptides in general to induce a substantial immune response. Rather, it is now widely believed that a major function of the carrier is to provide T-cell epitopes in close association with the B-cell epitopes on the short peptide which is statistically unlikely to contain both T-cell and B-cell sites recognized by the immune system of any given individual.

It may also be noted here that it has been shown that a T-cell epitope taken from one protein, in the form of a short peptide, may be combined with a short peptide comprising a B-cell epitope of another protein, to form a single peptide that induces a more complete and higher level immune response than either peptide alone.

More broadly, it is now widely accepted that the capability of any individual to mount any immune response

to a given epitope, as defined by a precise configuration of a small number of atoms, depends ultimately on the genetic make-up of the immune system genes which separately control the specificities of antigen recognition by B-cells and T-cells. Further, it is understood that the ability of a given B-cell epitope to induce cognate antibodies (i.e., antibodies which recognize that epitope) also depends upon the context within which that epitope is presented to the immune system, in terms of both associated T-cell epitopes and other B-cell epitopes. The latter sites may be "immunodominant" relative to the selected B-cell epitope of interest, that is, they may contend more effectively for the attention of the immune system than the selected B-cell epitope and thereby distract limited system resources from mounting the desired response to that selected epitope. In other words, B-cell epitopes that do not induce detectable antibodies in the presence of other, so-called immunodominant epitopes, which frequently occur in large polypeptides, often do induce significant levels of cognate antibodies when presented in a different context that lacks such immunodominant sites, on a short peptide, for example.

In conclusion, it is a further object of the present invention to exploit various consequences of the above noted characteristics of and distinctions between B-cell and T-cell epitopes, as well as methods for predicting and actually detecting amino acid sequences that serve as T-cell or B-cell epitopes. These will be discussed further below as needed in relation to the description of the present invention.

SUMMARY OF THE INVENTION

The recent molecular cloning, by the present inventor, of DNA segments encoding mouse ZP3 and ZP2 genes, and a major portion of a human ZP3 gene, and the subsequent characterization of the nucleotide sequences of their messenger RNAs (mRNAs) and the amino acid sequences encoded therein, have provided sufficient molecular detail of zona proteins to enable a new contraceptive approach. This strategy based on active alloimmunization with a zona pellucida polypeptide which includes an amino acid sequence that is selected to display at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm.

The complete nucleotide sequence of the mouse ZP3 messenger RNA and the amino acid sequence encoded thereby has been disclosed previously by the present inventor (M.J. Ringuette et al., 1988, Dev. Biol. **127**, 287-296, published June 13, 1988, the entire contents of which are hereby incorporated herein by reference).

The present inventor and associates have also reported (M. Chamberlin et al., 1987, abstract of a presentation at the twenty-seventh annual meeting of the American Society for Cell Biology, St. Louis, Missouri, November 16-20, J. Cell Biol. **105**, no. 4 part 2, 334A) that mouse genomic clones of the ZP3 gene and a human genomic DNA clone of the ZP3 gene have been isolated by virtue of their homology to the previously isolated murine ZP3 cDNAs. However, this abstract does not disclose specific nucleotide or amino acid sequences of any mouse or human DNA clone, nor does it even mention any concept of a contraceptive vaccine. Further, the mouse ZP2 cDNA sequences have not been disclosed previously.

Enabled by an oligonucleotide probe based on the short ZP3 cDNA sequence that was published by the present inventor (Ringuette et al., 1986, supra), and subsequent to publication of the complete mouse ZP3 cDNA sequence (M.J. Ringuette et al., 1988, Dev. Biol. **127**, 287-296), others have also reported isolation and sequences of genomic DNA clones of a mouse ZP3 gene and the amino acid sequence encoded therein (R. A. Kinloch et al., 1988, Proc. Natl. Acad. Sci. U.S.A. **85**, 6409-6413).

Whereas the prior art on contraceptive vaccines based on zona pellucida antigens has been and remains primarily focused on heteroimmunization, the present invention relates to contraceptive vaccines based on cloned zona pellucida genes and the strategy of alloimmunization with polypeptides including defined amino acid sequences that are selected for displaying epitopes to contraceptive antibodies. The advantages of this approach include the ability to produce and utilize those immunogens displaying the most effective B-cell epitopes for inhibition of fertilization regardless of whether or not they happen to be conserved in several species. Further, this vaccine strategy minimizes the likelihood of inducing antibodies with deleterious cross-reactivity with epitopes on molecules other than zona pellucida polypeptides. Ultimately, by reducing in the vaccine the number of B-cell epitopes that produce antibodies which, even though they bind to a zona pellucida antigen, do not block conception, this invention focuses the immune response to the vaccine on precisely those amino acids that are most critically situated to facilitated the contraceptive effect of antibodies. Further, by focusing on those epitopes that

are most useful for contraceptive purposes, the present invention minimizes potential interference with establishment of effective immunity to those critical contraceptive epitopes from extraneous epitopes that may be immunodominant to those critical sites and, therefore, may prevent an adequate contraceptive antibody response to them.

It is understood that in the practice of the present invention that epitopes may be used which happen to be conserved in the zona pellucida proteins of more than one species. However, in contrast to previous efforts to employ zona pellucida antigens in vaccines wherein the first concern has been to identify cross-reacting epitopes in heterologous antigens without initial regard for the functionality of such epitopes in inducing contraceptive antibodies, as described in some references cited herein above, it will be appreciated that use of conserved epitopes in the instant invention is entirely incidental to the goal of providing epitopes that are effective for inducing contraceptive antibodies in the particular target species intended for a given vaccine.

Accordingly, the present invention relates to a contraceptive vaccine for use in a mammalian female comprising a polypeptide which includes an amino acid sequence that is selected to display at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This contraceptive antibody epitope is an epitope for which there is a functional homolog displayed on a zona pellucida protein that originates from the species in which the said vaccine is used. The zona pellucida protein displaying the

functionally homologous epitope advantageously is either a ZP3 protein or a ZP2 protein or a ZP1 protein.

In other words, both the amino acid sequence of a polypeptide of this vaccine and a zona pellucida protein display epitopes which are functionally homologous in that they both are able to bind the same antibody that inhibits fertilization of an oocyte by a sperm. The fact that this vaccine polypeptide and a zona pellucida protein both display functionally homologous binding sites for the same antibody does not imply, however, that these binding sites are encoded by the same amino acid sequence in each instance, i.e., the polypeptides displaying the two epitopes are not necessary structurally homologous at the level of amino acid sequences encoding the epitopes.

By the phrase "originating from" it is meant that the zona pellucida protein is encoded in the genome of the species in which the said vaccine is used.

It will be understood from the foregoing Background that the nomenclature of zona pellucida proteins comprising ZP1, ZP2 and ZP3 has been defined in the mouse system and that other nomenclature or no nomenclature may be used in other mammalian systems. However, the present inventor has clearly demonstrated that the genes and mRNAs and, hence, the amino acid sequences of the major murine zona pellucida proteins (for example, the ZP3 and ZP2 proteins of the mouse) are highly conserved throughout diverse mammalian species (see below). In light of this high degree of structural similarity, a high degree of functional homology is also to expected in terms of the ability of homologous positions to serve as epitopes of contraceptive

antibodies. Accordingly, the terms "ZP3 protein", "ZP2 protein", and "ZP1 protein" contemplate not only the murine forms of these highly conserved zona pellucida proteins, but also the homologous counterparts of any other mammalian species, regardless of any other terminology by which such other proteins may be known in the art.

Contraceptive antibodies suitable for the practice of the present invention may be generated using zona pellucida antigens from natural sources, according to various published procedures. Alternatively, such antibodies may be produced advantageously by immunization with a polypeptide produced in a recombinant expression system comprising a DNA segment of the present invention. Various methods for identifying antibodies, including monoclonal antibodies, that inhibit the fertilization of an oocyte by a sperm have also been published (e.g., I. J. East et al., 1985, Dev. Biol. **109**, 268).

In the polypeptide of the vaccine of this invention, the amino acid sequence which displays an epitope for a contraceptive antibody may include all or part of the same amino acid sequence responsible for displaying the functionally identical epitope on a zona pellucida protein. In some cases, a single epitope for binding a given antibody comprises more than one contiguous amino acid sequence of a polypeptide (see discussion of "discontinuous epitope", below); accordingly, the present invention contemplates that the polypeptide of the vaccine may include at least one amino acid sequence of a zona pellucida protein that displays a functionally homologous epitope.

An amino acid sequence displaying an epitope for an available contraceptive antibody may be selected from all the sequences in a zona pellucida protein using a known contraceptive antibody. For example, a contraceptive antibody may be used to isolate a peptide displaying its epitope from a proteolytic digest of a zona pellucida protein by means of affinity chromatography methods that are well known in the art.

Alternatively, a DNA sequence encoding an amino acid sequence which displays an epitope for a contraceptive antibody may be isolated by standard genetic engineering approaches. These involve screening of clones of fragments of a gene for a zona pellucida protein for the ability to express an amino acid sequence that binds the contraceptive antibody.

Yet another way to identify an amino acid sequence that displays the epitope of a contraceptive antibody is to employ the well known strategy of chemical synthesis of every distinct peptide that could possibly display an antibody epitope. For instance, technology is commercially available for the rapid synthesis and antibody reactivity testing of all peptides of six amino acids that occur sequentially in the sequence of a protein and overlap by one amino acid. In the practice of the present invention, the sequences to be synthesized are determined advantageously from the nucleotide sequence of a cloned gene for a zona pellucida protein.

In another embodiment of this aspect of the present invention, the amino acid sequence that displays the epitope for a contraceptive antibody in the vaccine may be some type of analog of the amino acid sequence for that epitope on the zona pellucida protein.

One type of analog that this embodiment comprehends is a synthetic peptide known as a "mimotope" by H. M. Geysen, the inventor of the technology used to create such analogs, for which kits of materials are now commercially available. In a substantial number of cases, this synthetic epitope generation approach produces amino acid sequences that are functional analogs of known epitopes for a given antibody, and these analogs can induce other antibodies that recognize the same epitope as the original selected antibody. These analog sequences, however, usually do not contain the amino acids in the natural amino acid sequence that displays the selected epitope. Thus this type of analog sequence mimics a naturally occurring structure that displays an epitope, hence, the term "mimotope". An important feature of this particular aspect of this embodiment of the present invention is that it is not necessary to identify the natural amino acid sequence displaying the epitope of the desired contraceptive antibody; in fact, this method can produce small peptide analogs of natural epitopes comprising amino acids located in distinct positions of a protein that are separated by many amino acids (i.e., so-called "discontinuous epitopes" as opposed to those epitopes encoded by a single short continuous amino acid sequence).

In the term "analog", this aspect of the present invention also contemplates the application of well known principles of sequence conservation during the evolution of protein families to identify epitopes for contraceptive antibodies in a selected zona pellucida protein for which such antibodies are not yet available. If the an amino acid sequence of this zona pellucida

protein is highly homologous to that of related protein from another species, and if epitopes for such contraceptive antibodies have been defined in the sequence of this latter protein, then the general structural homology between the two proteins may be used to indicate those sequences in the selected protein that display epitopes for contraceptive antibodies that are analogous to those known for the second protein.

In other words, when two short, distinct amino acid sequences are known to occupy the same position in two proteins of substantially homologous structure (i.e., overall amino acid sequence and, consequently, three-dimensional conformation), then if one of the two sequences displays an epitope for an antibody with a particular biological effect, than the other sequence almost certainly displays epitopes for other antibodies with the same biological effect. According to this aspect of this invention, a known epitope for a contraceptive antibody is embodied by an amino acid sequence identified in a mouse ZP3 protein by screening cloned fragments of a cloned DNA for expression of suitable epitopes, and one analog of this amino acid sequence is embodied by the sequence of amino acids that occupies the homologous position in the human ZP3 protein. This human analog of a mouse ZP3 epitope (which also may be considered to be a "homologue" of that epitope), is to be incorporated into a vaccine for use in human beings, of course, according to the alloimmunization aspect of the present invention.

It is understood that chemically synthesized peptides may be used advantageously as polypeptides of the present invention, especially since the synthesis of

such peptides comprising 30 to 50 or even more amino acids can now be achieved on scales sufficient for vaccine purposes (in batches of 1 gram or more, for example). One such synthetic peptide is embodied by a mouse ZP3 peptide that is described below.

It should be particularly noted that the polypeptides of the present invention do not include idiotypic antibodies or large fragments of such antibodies, since the disadvantages of using such polypeptides to present epitopes of zona pellucida proteins has been discussed above in the Background in regard to prior art on such antibodies. However, the present invention does contemplate smaller polypeptides comprising mainly those amino acid sequences of such idiotypic antibodies that actually comprise the analog of the original zona pellucida protein epitope.

Further, as will be appreciated from the Background discussion of immunogenicity of polypeptides, the immunogenicity of polypeptides or peptides of the present invention in terms of raising higher titers of contraceptive antibodies with greater affinities for their epitopes, particularly such immunogenicity of small (synthetic) peptides, may be enhanced advantageously by covalent coupling to another polypeptide or peptide, especially to another amino acid sequence displaying a T-cell epitope.

In addition, it will be appreciated that, as is customary for vaccines, the polypeptides of the present invention will be delivered in a pharmacologically acceptable vehicle. Vaccines of the present invention may also advantageously comprise effective amounts of immunological adjuvants that are known to enhance the

immune response to immunogens in general, particularly adjuvants that enhance the immunogenicity of small synthetic peptides.

In another aspect, the present invention further relates to certain DNA segments that encode mouse ZP3 or ZP2 proteins and at least a portion of a human ZP3 protein. This invention also relates to cultures of recombinant cells containing a DNA segment of this invention, and to methods for the synthesis and isolation of polypeptides and peptides of this invention.

Finally, the present invention also relates to recombinant DNA molecules comprising a DNA segment of this invention and a vector. On particular embodiment of this aspect of this invention contemplates a contraceptive vaccine for use in a mammalian female comprising a vaccinia virus vector expressing a DNA sequence encoding at least a part of a zona pellucida protein.

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples and Figures included therein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 presents the complete nucleotide sequence and deduced amino acid sequence of the mouse ZP3 mRNA, as determined from sequencing cDNAs and genomic DNA clones.

Fig. 2 illustrates the high degree of structural homology between the mouse ZP3 sequences and the nucleotide and deduced amino acid sequences of a major portion of the human homolog of the mouse ZP3 protein.

Fig 3. presents the complete nucleotide sequence and deduced amino acid sequence of the mouse ZP2 mRNA, as

determined from sequencing cDNAs.

Fig. 4. outlines the definition of a mouse ZP3 epitope for a contraceptive antibody comprising a sequence of 7 amino acids.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention relates in part to DNA segments having sequences that encode ZP3 proteins. One embodiment of this aspect of the invention is a cDNA clone (e.g., pZP3.1 or pZP3.2) that encodes at least a portion of the complete nucleotide sequence of the mouse ZP3 mRNA and the amino acid sequence encoded thereby (see Figure 1) which has been determined by the present inventor, as described in Example 1, below, and has been published (M.J. Ringuette et al., 1988, Dev. Biol. **127**, 287-296). In Figure 1, the initiation and termination codons are boxed and the polyadenylation signal is overlined. The single 1272-nucleotide (nt) open reading frame is translated into the ZP3 polypeptide in the second line. The proposed signal peptide is indicated by a wavy line and the signal peptidase cut site by an arrow. The deduced amino acid sequence corresponding to an experimentally determined amino acid sequence of an internal ZP3 peptide is underlined with a dashed line and the six potential N-linked glycosylation sites (Asn-X-Thr/Ser) are underlined with a solid line. The map positions of the two synthetic oligonucleotides used for substantiating the size of the mRNA are indicated by dots.

In brief, the ZP3 mRNA contains relatively short 5' and 3' untranslated regions and an open reading frame sufficient to code for a precursor protein of 46,307 Da. Sequences similar to the mouse ZP3 mRNA are present in

the genome of a variety of mammals and that they express poly(A)$^+$ RNA transcripts that are indistinguishable in length from mouse ZP3 mRNA.

The present invention also relates to DNA segments with sequences that comprise mouse genomic clones of the ZP3 gene and a human genomic DNA clone of the ZP3 gene comprising at least five of the eight exons. These genomic clones have been isolated using standard genetic engineering approaches well known in the art by virtue of their homology to the previously isolated murine ZP3 cDNAs. Figure 2 presents a comparison between mouse ZP3 sequences and the nucleotide and deduced amino acid sequences of a portion of the human homolog of the mouse ZP3 protein encompassing five of the eight exons of the human gene. In Figure 2, panel A is a comparison of the nucleotide sequences and panel B compares corresponding amino acid sequences. In each panel, the top line depicts human sequences and the bottom, those of mouse. A gap in one sequence or the other indicates an insertion or deletion of sequences in one species relative to the other. A vertical bar from a nucleotide or amino acid in the top line to the corresponding one in the bottom line indicates exact identity of the two sequences at that position. A pair of dots between corresponding amino acids in the two sequences indicates chemical similarity of the two sequences, while a single dot indicates evolutionary similarity (i.e., a pair of amino acids that are frequently substituted in different members of protein families that are well conserved across many species).

The data in Fig. 2 clearly show the high homology of the mouse and human ZP3 sequences, as would

be expected from the extensive nucleic acid hybridization observed between mouse ZP3 cDNA and genomic DNAs from a variety of other mammalian species (see Example 1). From this structural homology data, and further standard analyses thereof (e.g., predictions of secondary structure, hydropathicity, or surface accessibility), it would be apparent to one of average skill in the art of protein structure and immunology that the mouse and human ZP3 proteins must also exhibit throughout their entire sequences an extremely high level of functional homology with respect to locations that are able to induce and bind contraceptive antibodies. Thus, although epitopes for contraceptive antibodies on each protein may comprise short amino acid sequences which are not precisely conserved between the two proteins, the human sequences corresponding to such epitopes on the mouse protein are also expected to induce functionally homologous antibodies, even though the mouse and human antibodies might only recognize their respective alloantigens.

Further, the present invention relates to DNAs encoding mouse ZP2 cDNA sequences comprising at least a portion of the complete nucleotide and deduced amino acid sequences of the mRNA for the mouse ZP2 protein, as depicted in Figure 3.

It will be obvious, of course, to one of ordinary skill in the art of genetic engineering, that the above ZP3 and ZP2 sequences may vary slightly (i.e., be mutated) from one inbred mouse strain to another, or from one individual in an outbred population (e.g., one human being) to another, without materially affecting the immunological character of the corresponding zona pellucida protein and, therefore, without departing from

the scope of the DNAs of the present invention as conveyed, for example, by the use of the terms "the mouse ZP3 protein" or "the human ZP3 protein".

The DNA segments of the present invention variously enable development of different embodiments of the main aspect of the present invention, namely contraceptive vaccines for use in a mammalian female comprising a polypeptide which includes an amino acid sequence that is selected to display at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This contraceptive antibody epitope is an epitope for which there is a functional homolog displayed on a zona pellucida protein that originates from the species in which the said vaccine is used. The zona pellucida protein displaying the functionally homologous epitope advantageously is either a ZP3 protein or a ZP2 protein or a ZP1 protein.

In a principal embodiment of this aspect of this invention, a contraceptive antibody epitope that is displayed on the mouse ZP3 protein by a sequence of seven amino acids has been identified, and a synthetic peptide vaccine displaying that epitope has been shown to provide effective contraception in the mouse which is a convenient model system for identifying and testing such epitopes. Figure 4 outlines the definition of this mouse ZP3 epitope for a contraceptive antibody, which is described in further detail in Example 2, below. In Figure 4, panel A shows a schematic representation of the 1317-nt ZP3 mRNA. The single 1272-nt open reading frame is indicated by an open bar. The lines below the mRNA represent 8 positive cDNA clones isolated from a ZP3 epitope library in λgt11 by an anti-ZP3 monoclonal

antibody (I. J. East et al., 1985, Dev. Biol. **109**, 268). The clones are aligned on the ZP3 mRNA and the hatched bar indicates the sequence common to all positive clones. Three clones (*) define the 5' and 3' ends of the epitope. Panel B shows the DNA sequence of the overlapping region among the eight positive clones, and the corresponding amino acid sequences are shown in capital letters. The one additional C-terminal and eight additional N-terminal amino acids (lower case letters) shown flanking the epitope were included in the peptide used for immunization, although the sequences of the epitope clones clearly indicates that none of these amino acids is needed for antibody binding. Panel C depicts the hydrophilicity of the deduced 424 amino acid mouse ZP3 protein, plotted according to the Hopp and Woods algorithm using a 7 residue moving average. Horizontal filled-in bars beneath the hydrophilicity plot indicate amphipathic α helical segments predicted by the algorithm of Margalit et al. using an eleven residue moving average. The speckled vertical bar represents the 16 amino acid peptide shown in panel B which was used to immunize experimental animals.

In brief, a cDNA encoding ZP3 was randomly fragmented and 200-500 bp fragments were cloned into the expression vector gt11. This epitope library was screened with the aforementioned contraceptive antibody and the positive clones were used to map the seven amino acid epitope recognized by the antibody. Female mice were immunized with a synthetic peptide containing the epitope and the resultant circulating anti-ZP3 antibodies bound to the oocytes of immunized animals producing long-lasting contraception.

Of course, it would be obvious to one skilled in the art of synthetic peptide vaccines that a shorter portion of the 16 amino acid sequence that displays the ZP3 epitope described in Example 2 might also be an effective peptide of the present invention, (especially sequences consisting essentially of five or six of the seven amino acids encoded by the common sequence of the epitope clones, and sequences excluding the first eight amino acids or the last Gln, all of which were added for convenience without evidence of their necessity for the functioning of the synthetic peptide as a vaccine). It will be recognized, also, that certain analogs (e.g., those sequences with ends that are chemically modified to neutralize charges as is frequently practiced in the art) might provide effective peptides for the practice of the present invention.

The reversibility of the contraceptive effect, described in Example 2, can be accounted for by resting oocytes entering into the growth phase and synthesizing a zona pellucida in the presence of low-levels of circulating anti-zona antibodies which appear to decline after immunization with the vaccine is terminated. When ovulated, these oocytes would be coated lightly, if at all, with anti-zona antibodies and would, therefore, be capable of being fertilized.

These studies have demonstrated that repeated immunization of female mice with a mouse ZP3 peptide-KLH conjugate results in longterm infertility in the majority of cases. The production of anti-zona pellucida antibodies occurs despite the fact that the zona peptide is a self antigen (alloantigen). Immune tolerance has been postulated to occur in the neonatal period of

development and involves both the functional inactivation of B cells and the deletion of T cells which recognize self antigens. The lack of detectable zona proteins in the ovary until 2-3 days after birth, or their inaccessibility to the developing immune system, may account for the continued presence of lymphocytes capable of recognizing at least one ZP3 epitope.

In regard to the eventual reversibility of the contraceptive immunization, it is curious that having mounted an immunological response against the ZP3 peptide-KLH conjugate, the immune system does not continue to be stimulated by the endogenous ZP3 protein. The following hypotheses may account for this phenomenon in whole or in part, and, therefore, aid in understanding the present invention; but these theoretical explanations should not be construed to limit the scope of the present invention in any way. Nevertheless, it may be speculated that one or more of the following may be involved in the reversibility of the contraceptive immunization: 1) The localization of the zona proteins uniquely to the ovary coupled with the lack of capillaries beyond the basement membrane surrounding the follicles, may physically preclude lymphocytes from interacting with and being stimulated by the zona pellucida; 2) The 16 amino acid ZP3 peptide portion of the immunogen provides a B-cell epitope but may not contain T-cell epitopes (which may, instead, be provided by the KLH moiety) to stimulate helper T-cell functions. Thus, the endogenous ZP3 protein, although containing the same ZP3 peptide, would not contain the T-cell epitopes of the carrier protein that, according to this hypothesis, could be important for mounting an anti-ZP3 peptide response; 3) The ovary

may be part of an immunologically protected region and mechanisms that suppress the immunological rejection of the embryo (which contains paternal and, thus, foreign antigens) also function in the ovary.

It is particularly important to note that immunization with the ZP3 peptide vaccine did not result in either structural or functional abnormalities of the mouse ovary (viz, normal histology and the ability of vaccinated females to subsequently have litters). In this regard, of course, the use of a synthetic ZP3 peptide as a vaccine precludes any possible minor contamination with other ovarian immunogens. In addition, the physical barrier of the follicular basement membrane and the extra-cellular site of the zona protein may contribute to the absence of an immunocytotoxic response in the ovary.

The ZP3 epitope recognized by the monoclonal antibody used to develop this vaccine is not detected immunologically in hamster, guinea pig, cat or dog ovaries. Thus, this ZP3 peptide reported in the current study would not be expected to act as a contraceptive in other mammalian species, including human beings, although the ability of this antibody to bind to the human ZP3 protein has not been tested. However, strategy of the present invention of using vaccination with "self" zona peptides can be applied to other species by taking advantage of the highly conserved nature of the zona genes among mammals which was described in Example 1. Accordingly, as noted above, the human homologue of the mouse ZP3 gene has been identified and the sequence of at least six of the eight exons is greater than 80% similar to that of the mouse ZP3 gene. As discussed above, this

high degree of structural homology indicates comparable functional homology in relation to epitopes for contraceptive antibodies.

Accordingly, using the deduced primary amino acid sequence of the human ZP3 protein, by the practice of the present invention without undue experimentation, it is believed that one of ordinary skill in the art of polypeptide structure and immunology can identify in the human or other mammalian ZP3 protein the region homologous to the mouse ZP3 peptide described herein. Alternatively, one of such skill may use computer algorithms to predict additional epitopes which may be potential immunogens [T.P. Hopp and K.R. Woods, Proc. Natl. Acad. Sci. USA **78**, 3824 (1981); H. Maragalit, J.L. Spouge, J.L. Cornette, K.B. Cease, C. Delisi, J.A. Berzofsky, J. Immunol. **138**, 2213 (1987); J.B. Rothbard and W.R. Taylor, EMBO J. **7**, 93 (1988)], or test a large array of peptides representative of the polypeptide chain for epitopes of contraceptive antibodies using well known methods [H.M. Geysen, R.H. Meloen and S.J. Barteling, Proc. Natl. Acad. Sci. USA **81**, 3998 (1984); R.A. Houghten, Proc. Natl. Acad. Sci. USA **82**, 5131 (1985); H.M. Geysen, J.A. Tainer, S.J. Rodda, T.J. Mason, H. Alexander, E.D. Getzoff and R.A. Lerner, Science **235**, 1184 (1987); E. Norrby, M.A. Mufson, H. Alexander, R.A. Houghten and R.A. Lerner, Proc. Natl. Acad. Sci. USA **84**, 6572 (1987)].

Further, as noted previously, one skilled in the art of synthetic peptide vaccines can also develop "mimotopes" of epitopes to available contraceptive antibodies. According to this approach, first, the ability of any desired antibody to bind to essentially

every possible sequence of two amino acids that naturally appear in proteins is tested. Upon identification of a pair of amino acids with detectable binding of the antibody, the sequence surrounding those two amino acids is progressively and systematically varied, by the inclusion of each of the naturally occurring amino acids as well as some amino acids not found in natural proteins, until continued testing of antibody binding identifies a short peptide displaying an epitope with sufficient affinity for the selected antibody to be used for the desired purpose.

Thus, the approach of this invention of alloimmunization with epitopes of zona proteins is expected to have wide application in the design of future contraceptive vaccines for the control of mammalian populations.

Example 1. Characterization of nucleic acid and amino acid sequences of ZP3 proteins.

Size of the ZP3 mRNA. Previous studies (Ringuette et al., 1986, supra) reported the isolation of two overlapping cDNAs coding for mouse ZP3 with a total length of 1.3 kb. Present Northern blot analyses indicate that the ZP3 gene is transcribed as a 1.5- to 1.6-kb polyadenylated mRNA. Repeated rescreenings of the original λ gt11 ovarian library were unable to identify longer ZP3 cDNA clones. However, the nucleic acid sequence of the two available clones lacked an initiation codon for the single open reading frame. Therefore, to determine the size of the full-length message, a 20-nt oligonucleotide corresponding to map position 154-173 was synthesized and used for primer extension studies. [For consistency, map positions refer to the full-length ZP3

transcript, the sequence of which was ultimately determined from two cDNA clones and a genomic clone.]

Labeled 20-mer was annealed to ovarian poly(A)$^+$ RNA at 65°C (T_d, -5°C). Although these conditions assured high hybridization specificity, they inhibited M-MLV reverse transcriptase. Therefore, just prior to reverse transcription, the temperature was decreased to 37°C and buffer conditions were optimized for reverse transcriptase activity. However, the decrease in temperature resulted in nonspecific binding of the 20-mer to noncomplementary RNA sequences. To circumvent these problems, unlabeled primer was added just prior to decreasing the temperature of the annealing reactions. A 66-fold excess of unlabeled 20-mer was effective in eliminating signals obtained from non-target site primer extension products. The results showed a 170- to 175 nt extension product specific to ovarian poly(A)+ RNA that is not seen when liver RNA is used as a template.

To substantiate further the size of ZP3 mRNA, oligonucleotide-directed cleavage of messenger RNA by ribonuclease II was carried out. Two synthetic oligonucleotides, one corresponding to map position 154-173 and the other to map position 287-303, were independently annealed to mouse ovarian poly(A)$^+$ RNA, digested with E. coli ribonuclease H, and probed with a ^{32}P-labeled ZP3 cDNA which extends from map position 47 to 1275. Cleavage occurs only at the region where the oligonucleotide hybridizes to the RNA and results in a decrease in the observed size of the ZP3 mRNA by approximately 160 and 300 nt, respectively. The corresponding smaller bands (approximately 155 and 285 bases) were not detected despite repeated probing and

long exposures. Ribonuclease II, in the absence of an oligonucleotide, had no effect on the molecular weight of ZP3. These results support the primer extension data which indicates that the ZP3 mRNA extends an additional 46 nt more 5' than the longer of the two cDNA clones.

Nucleic Acid Sequences and Deduced Amino Acid Sequence of Mouse ZP3. The nucleic acid sequence of the two cDNAs coding for ZP3 was determined, and a recently isolated genomic clone of mouse ZP3 was used to define the remaining 46 nucleic acid residues not represented in the cDNA clones (Fig. 1). The ZP3 transcript is 1317 bp long and the cDNA clones represent 97% of a full-length copy. The transcript has a relatively short 29-nt 5' untranslated region followed by a single open reading frame of 1272 nt which commences with an ATG embedded in the ANNATG motif associated with vertebrate initiator codons. The 3' untranslated region is also short (16 nt) and the TAA termination codon is a part of the canonical AATAAA polyadenylation signal which precedes the start of the poly(A) tail by 12 nt.

The open reading frame translates into a protein (see Fig. 1) with a molecular weight of 46,307 Da which consists of 124 amino acids (9% acidic, 7.3% basic, 7.5% aromatic, and 31.4% hydrophobic). It contains the 20-amino acid sequence which was previously compared to the sequence of an internal ZP3 peptide and used to confirm the identity of the ZP3 cDNA clones. The National Biomedical Research Foundation Protein Data Bank was searched for sequences similar to those of the ZP3 protein using the FASTP computer program. Several proteins were shown to have short regions of amino acid sequence similar to those of ZP3, but the similarities

were of borderline statistical significance and the identified proteins had no apparent biological correlation with ZP3. The ZP3 protein translated from the nucleic acid sequence contains six Asn-X-Ser/Thr sequences, each representing a potential N-linked glycosylation site. The asparagine at amino acid position 273 has previously been shown to be derivatized (Ringuette et al., 1986, supra) and presumably represents one of the glycosylation sites; the status of the other sites remains unknown. The charged amino acids are well-distributed along the ZP3 protein except in the two major regions of hydropathicity found at the amino and carboxyl termini where they are absent. There are six regions which, theoretically, have a high degree of α-helical structure and two of these regions are in the terminal hydrophobic regions.

The first 17 residues of the amino terminus of the full-length protein are quite hydrophobic and the first 11 can be formed into an α-helical structure followed by a β-turn. Using the sliding window/matrix scoring method of von Heijne, we have identified a potential peptidase cut site after the 22nd amino acid. The resultant secreted protein would have a molecular weight of 43,943 Da, consistent with the reported 44,000 Da molecular weight of the ZP3 core protein. Despite making two attempts, the N-terminal amino acid sequence of the secreted ZP3 protein could not be identified by micro gas-phase sequencing, suggesting that it may be blocked by chemical modification. Near the carboxyl terminus of the deduced ZP3 polypeptide, there is a second hydrophobic region of 26 amino acids, the function of which is unknown. It is intriguing that this region

contains several 19-residue-long segments with hydropathicity indices of 2.2, which is well in excess of the 1.09 0.22 average associated with internal globular protein domains. Normally such hydrophobic domains are seen in membrane-spanning regions, although the mature ZP3 protein is clearly an extracellular matrix protein. This hydrophobic region may play a role in interactions with other zona proteins or with the oocyte's membrane.

Conservation among Mammals. It has been reported that there are genes with sequences similar to mouse ZP3 in the genomes of a variety of mammalian species (Ringuette et al., 1986, supra). These data presumably reflect the common structure and function of the extracellular zona pellucida of different mammalian species. These studies have been extended by using the mouse cDNA to probe genomic DNA isolated from animals spanning the evolutionary tree.

Genomically equivalent amounts of DNA from human, mouse, rat, and chicken were digested with BamHI, electrophoresed on 0.8% agarose gels, blotted onto nitrocellulose, and probed with ^{32}P-labeled insert from pZP3.2 cDNA. After washing under conditions (22°C below T_m) that would detect sequences having similarities greater than 78%, signals were detected in restriction fragments of human (6.2 kbp), mouse (9.8 and 6.8 kbp), and rat (7.9, 2.65, 2.25, and 1.7 kbp) DNA. Surprisingly, a weak but reproducible signal was observed with chicken genomic DNA cut with either BamHI (7.9 kbp) or HindIII (5.9 and 0.9 kbp). A computer search of The Genetic Sequence Data Bank (Gen-Bank) did not identify any sequences similarities among chicken DNA sequences except for a short segment of the coding strand of ZP3

cDNA (map position 605-667) which was 73% similar to the noncoding strand of a potential calcium-binding domain of chicken calcium protease. However, no signal was observed when hybridizing a ^{32}P-labeled ZP3 cDNA insert to a Northern blot containing brain, liver, oviductal, and ovarian RNA isolated from chickens. It, therefore, appears unlikely that the signal obtained in the Southern blot of genomic chicken DNA corresponds to a transcribed chicken oocyte gene homologous to mouse ZP3 cDNA.

Extracellular coatings surround the oocytes of a number of nonmammalian species. Therefore, it was of interest to determine if sequences similar to ZP3 were present in a variety of organisms. Approximate genomic equivalents of DNA from X. laevis, Rainbow trout, S. purpuratus, D. melanogaster, and, for evolutionary interest, S. cerevisiae were digested with BamHI and hybridized with ^{32}P-labeled pZP3.2 cDNA insert. Under wash conditions (44°C below T_m) where similarities as little as 56% should be detected, cross-hybridization to nonmammalian species was eliminated. Thus, the ZP3 gene appears to be found exclusively in mammalian genomes.

To determine if genomic loci similar in sequence to ZP3 from other mammals were expressed, ovarian poly(A)$^+$ RNA isolated from a variety of mammalian species (mouse, rat, rabbit, dog, and cow) was probed with ^{32}P-labeled pZP3.2 cDNA insert. All ovarian tissues contained ZP3 transcripts. Furthermore, Northern blot analysis of ZP3 transcripts from three species, mouse, rat, and rabbit indicate that the ZP3 transcripts have similar molecular weights. This observation was substantiated by detecting only one band on the Northern blot after mixing mouse/rat or mouse/rabbit RNA.

Example 2. A contraceptive vaccine comprising a synthetic peptide with a mouse ZP3 epitope.

Generation and screening of an epitope library from a ZP3 cDNA. A 1.0 kb cDNA known to contain the epitope recognized by the anti-ZP3 monoclonal antibody (Ringuette et al., 1986, supra) was cut into random fragments which were size selected (200 bp) and cloned into the gt11 expression vector. More specifically, the cDNA insert of pZP3.1 was digested with DNase in the presence of 15 mM $MgCl_2$ and 200 bp size selected fragments [V. Mehra, D. Sweetwer and R.A. Young, Proc. Natl. Acad. Sci. USA 83, 7013 (1986)] were ligated into Lambda ZAP (Strategene). E. coli BB4 cells were infected with the un-amplified epitope library and screened [Ringuette et al., 1986, supra] with an anti-ZP3 monoclonal antibody [I. J. East et al., 1985, Dev. Biol. **109**, 268]. Positive clones were plaque purified and the sequence of the insert DNA was determined from isolated plasmid DNA [F. Sanger, S. Nicklen and A.R. Coulson, Proc. Natl. Acad. Sci. USA **74**, 5463 (1977)].

A synthetic peptide displaying an epitope for a contraceptive antibody. The nucleic acid sequence of the cDNA inserts from 8 positive clones was determined (Fig. 4A). The 24 nucleotides common to the eight clones code for a seven amino acid peptide which must contain the epitope recognized by the antibody (Fig. 4B). The peptide represents amino acids 336-342 which is immediately adjacent to the most hydrophilic portion of ZP3 and partially overlaps a region which contains an amphipathic α-helix (Fig. 4C). Both of these attributes have been associated with immunodominant epitopes.

A 16 amino acid peptide (ZP3 amino acids 328-343) containing the epitope (NH_2-cys-ser-asn-ser-ser-ser-ser-gln-PHE-GLN-ILE-HIS-GLY-PRO-ARG-gln-COOH) was synthesized and coupled via the N-terminal cysteine to keyhole limpet hemocyanin (KLH).

The sixteen amino acid peptide was synthesized [Merrifield, R.B., J. Amer. Soc., **85**, 2149, (1963)] on a Model 430A, Applied Biosystems Solid Phase Synthesizer, deprotected and released from the phenylacetamidomethyl resin with anhydrous hydrogen fluoride containing 10% anisole and 10% thiophenol at 0°C for 2 hr. The crude peptide was purified by HPLC on a Vydac C4 column and conjugated to keyhole limpet hemocyanin by coupling the amino terminal cysteine to KLH through a maleimido linkage [Lerner, R.A. et al., Proc. Natl. Acad. Sci. USA, **78**, 3403, (1981)].

Immunogenicity of the synthetic peptide vaccine. Sixteen NIH random bred Swiss mice were immunized intraperitoneally with 100 μg of the ZP3 peptide-KLH conjugate (1 mg/ml) in an equal volume of complete Freund's adjuvant and then boosted at 10-14 day intervals with 100 μg of conjugated peptide in incomplete Freund's adjuvant. Circulating anti-zona pellucida antibodies were detected using solubilized whole zona in an ELISA. Flexible ELISA plates were coated with purified, acid solubilized zona [J.D. Bleil and P.M. Wassarman, J. Cell Biol. **102**, 1363 (1986)] at 100 ng per well, blocked with 1% bovine serum albumin in Tris HCl, pH 7.5, 0.15 M NaCl (TBS), and incubated with sera diluted 1:10^4 in the same. The plates were washed several times with TBS/1% Tween-20, incubated with horse radish peroxidase (HRP) conjugated goat anti-mouse antibody, washed as before,

and developed using a Horseradish Peroxidase Substrate Kit (Bio-Rad). The response was quantified by measuring absorbance at 414nm.

A plateau level of the average response as reached after five immunizations. It should be noted that there was variation of the amount of circulating anti-zona pellucida antibodies among the animals with the difference between the high and low responders being almost sixfold. Control animals were immunized with KLH alone using an identical regimen and had no detectable circulating anti-zona antibodies.

The reactivity of sera from immunized animals with individual zona proteins was analyzed using Western blots of purified zonae separated by SDS-PAGE. Isolated mouse zona were acid solubilized and separated by SDS-PAGE using 10% acrylamide [U.K. Laemmli, Nature **227**, 680 (1970)]. Proteins were transferred to nitrocellulose [W.N. Burnette, Analyt. Biochem. **112**, 195 (1980)] and the filters soaked in TBS/1% BSA. Sera or antibodies were diluted in TBS/1% BSA/0.1% Tween and individual lanes were probed with: pre-immune sera diluted 1:50; immune sera from KLH immunized mice diluted 1:50; immune sera from ZP3 peptide-KLH immunized mice diluted 1:50; rat anti-mouse ZP3 monoclonal antibody [8] diluted 1:50; and rabbit anti-mouse zona pellucida polyclonal antisera [East et al. 1985, supra] diluted 1:50. Filters were washed in TBS/0.1% Tween and incubated with HRP-labeled second antibody of the appropriate specificity (Jackson Immunoresearch) diluted 1:1000 in TBS/BSA/Tween. Nitrocellulose-bound antibodies were visualized using 4-chloro-1-naphthol.

Sera from animals immunized with the ZP3 peptide-KLH conjugate reacted with a single zona protein which co-migrated with ZP3. No reaction with any of the zona proteins was detected with pre-immune or control sera.

To determine whether anti-peptide antibodies recognize zona in its native state as well as in acid-solubilized and SDS-denatured preparations, sera from experimental and control animals were used to stain unfixed frozen sections of mouse ovary. Ovaries were removed and immediately frozen in Tissue-Tek O.C.T. Compound (Lab-Tek Products) on dry ice. Five μm sections were mounted on gelatin coated slides, treated with 1% BSA in PBS for 15 min at 20°C and rinsed in PBS. Sections were treated for one hour with undiluted serum from immunized mice, rinsed in PBS and stained for 30 min at 20°C with FITC-conjugated goat anti-mouse IgG (Jackson ImunoResearch Laboratories) diluted 1:50 in PBS/BSA. Sections were rinsed with PBS, mounted in Fluormount-S (FisherBiotech) and photographed using Ektachrome 200 film.

Using a fluorescein-conjugated second antibody, mouse antibodies from experimental mice were detected binding to the zonae surrounding developing oocytes, indicating that the circulating anti-zona antibodies are capable of binding native ZP3 protein. There was no detectable fluorescence of sections stained with sera from control mice.

To determine if the circulating anti-ZP3 antibodies were of sufficient titer to bind to the zonae surrounding growing oocytes of the experimental mice, plastic embedded sections of ovaries isolated from four

females immunized with ZP3-KLH conjugate were stained with horse radish peroxidase (HRP) conjugated anti-mouse antibody. Dissected ovaries were fixed for one hour in 1% glutaraldehyde, rinsed in PBS and embedded in JB4 plastic. Endogenous antibody was detected in 4 μm sections using an antimouse streptavidin-HRP kit (Zymed).

Mouse anti-zona pellucida antibodies were observed coating the zonae of the oocytes in the sections examined. There were no detectable anti-zona antibodies in ovaries isolated from four control (KLH alone injected) mice. The ovarian sections of both the treated and control animals contained only normal follicles and cell types with no evidence of inflammation or cellular cytotoxicity. The antisera of the ZP3-KLH immunized animals did not react with other mouse tissue including brain, liver, spleen, kidney, heart, lung, intestine, testis or muscle (data not shown) which indicates that immunization with the peptide conjugate elicits a response that is specific for the zona pellucida.

Effectiveness of the synthetic peptide vaccine for contraception. The fertility of the remaining 12 experimental and 12 control mice was tested by mating them continuously with proven males. Two weeks after the last immunization, proven males were individually and continuously caged with experimental and control mice at a ratio of 1:1. The percentage of animals having given birth to a litter versus the duration of continuous mating was compared for animals injected with ZP3 peptide-KLH and KLH alone. The titer of anti-ZP antibodies of three groups of ZP3 peptide-KLH immunized mice at the beginning of the mating period were averaged and, in order of increasing average titers, were as

follows: group 1, gave birth within 1 month (3 animals); group 2, gave birth between 4 and 7 months (3 animals); and group 3, did not give birth to litters within the 9 month study (6 animals).

In summary, all of the control (KLH alone injected) mice gave birth to litters within three and a half weeks of the introduction of males. Three of the experimental, ZP3 peptide-KLH injected mice also gave birth within this period. These mice were among those that had the lowest titers (<0.2 A_{414} units) of anti-zona antibodies prior to mating. In the remainder of the experimental mice a contraceptive effect was observed that lasted between 16 and 36 weeks at which time the study was terminated. Three of these animals gave birth to litters after 16 to 24 weeks and had intermediate anti-zona antibody titers. The remaining animals which remained infertile for the duration of the study had the highest initial titers and even 9 months after the last immunization had detectable circulating antizona antibodies.

The litter sizes of the ZP3-KLH treated animals which eventually became fertile ranged from 1-5 pups (average 2.8) whereas those treated with KLH alone had litters of 1-9 pups (average 5.2). Both groups had fewer than the normal 7-14 pups (average 10) which may be due, in part, to the adverse effects of intraperitoneal administration of Freund's adjuvant on fecundity. In addition, the smaller litters of the KLH-ZP3 treated animals could be accounted for by the observed persistent low levels of circulating anti-zona antibodies some of which were detected binding to the zonae surrounding their intra-ovarian oocytes. Despite the presence of

these low levels of anti-zona antibodies, these animals, when re-mated, gave birth to litters within three and a half weeks.

* * * * * * * * * * *

For purposes of completing the background description and present disclosure, each of the published articles, patents and patent applications heretofore identified in this specification are hereby incorporated by reference into the specification.

The foregoing invention has been described in some detail for purposes of clarity and understanding. It will also be obvious that various combinations in form and detail can be made without departing from the scope of the invention.

WHAT IS CLAIMED IS:

1. A contraceptive vaccine for use in a mammalian female comprising a polypeptide which includes an amino acid sequence that is selected to display at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm;

a functional homolog of said epitope being displayed on a zona pellucida protein; and

said zona pellucida protein originating from the species in which said vaccine is used;

said vaccine further comprising a pharmacologically acceptable vehicle.

2. The contraceptive vaccine according to claim 1 wherein said zona pellucida protein is selected from the group consisting of: the ZP3 protein, the ZP2 protein, and the ZP1 protein.

3. The contraceptive vaccine according to claim 1 wherein said amino acid sequence that displays said epitope includes at least one amino acid sequence of said zona pellucida protein.

4. The contraceptive vaccine according to claim 1 wherein said amino acid sequence which displays said epitope includes an analog of at least one amino acid sequence of said zona pellucida protein.

5. The contraceptive vaccine according to claim 4 wherein said amino acid sequence which displays said epitope includes an analog of at least one amino acid sequence of the mouse ZP3 protein.

6. The contraceptive vaccine according to claim 4 wherein said amino acid sequence which displays said epitope includes an analog of at least one amino acid sequence of the mouse ZP2 protein.

7. The contraceptive vaccine according to claim 1, further comprising a synthetic peptide that displays the said epitope.

8. The contraceptive vaccine according to claim 7 wherein said synthetic peptide includes the mouse ZP3 amino acid sequence: Phe-Gln-Ile-His-Gly-Pro-Arg-Gln.

9. The contraceptive vaccine according to claim 8 wherein said synthetic peptide further includes the mouse ZP3 amino acid sequence: Cys-Ser-Asn-Ser-Ser-Ser-Ser-Gln.

10. The contraceptive vaccine according to claim 7 wherein said synthetic peptide includes an analog of the mouse ZP3 amino acid sequence: Phe-Gln-Ile-His-Gly-Pro-Arg.

11. The contraceptive vaccine according to claim 8 wherein said synthetic peptide further includes an analog of the mouse ZP3 amino acid sequence: Cys-Ser-Asn-Ser-Ser-Ser-Ser-Gln.

12. The contraceptive vaccine according to claim 10, wherein said analog comprises an amino acid sequence that is included in the homologous position in a ZP3 protein from any mammal other than a mouse.

13. The contraceptive vaccine according to claim 1, wherein said mammalian female in which said vaccine is used is selected from the group consisting of: a cat, a dog, a pig, a cow, and a woman.

14. The contraceptive vaccine according to claim 1, further comprising amino acid sequences that are effective for enhancing the contraceptive antibody response to said epitope in the species in which said vaccine is used.

15. The contraceptive vaccine according to claim 14, said sequences for enhancing said antibody response to said epitope being contained in the polypeptide that displays said epitope.

16. The contraceptive vaccine according to claim 14, said sequences for enhancing said antibody response to said epitope being contained in a second polypeptide that is covalently linked to the polypeptide that displays said epitope.

17. The contraceptive vaccine according to claim 14, wherein said amino acid sequences for enhancing said antibody response to said epitope further comprising at least one T-cell epitope.

18. The contraceptive vaccine according to claim 1, further comprising an effective amount of an adjuvant.

19. A DNA segment encoding at least a portion of the mouse ZP3 protein.

20. A DNA segment encoding at least a portion of the mouse ZP2 protein.

21. A DNA segment encoding at least a portion of the human ZP3 protein.

22. A recombinant DNA molecule comprising a DNA segment according to claim 21 and a vector.

23. A culture of cells transformed with a DNA segment according to claim 21.

24. A method of producing at least a portion of a human ZP3 protein comprising culturing cells according to claim 23 under conditions such that said protein is produced and isolating said protein from said cells.

25. An antibody specific for a protein having the amino acid sequence of at least a portion of the

human ZP3 protein.

26. An antibody according to claim 25, wherein said antibody inhibits fertilization of a human oocyte by a sperm.

FIG. 1-1

```
                                              30
 CT GAG CCC AGC TGT ACT CCA GGC GGG ACC ATG GCG TCA AGC TAT
                                        Met Ala Ser Ser Tyr

                      60
TTC CTC TTC CTT TGT CTC CTG CTG TGT GGA GGC CCC GAG CTG TGC
Phe Leu Phe Leu Cys Leu Leu Leu Cys Gly Gly Pro Glu Leu Cys

                                          120
AAT TCC CAG ACT CTG TGG CTT TTG CCG GGT GGA ACT CCC ACC CCA
Asn Ser Gln Thr Leu Trp Leu Leu Pro Gly Gly Thr Pro Thr Pro

                      150
GTG GGG TCC TCA TCA CCT GTG AAG GTG GAG TGT CTG GAA GCT GAA
Val Gly Ser Ser Ser Pro Val Lys Val Glu Cys Leu Glu Ala Glu

                                          210
CTA GTG GTG ACT GTC AGT AGA GAC CTT TTT GGC ACG GGG AAG CTG
Leu Val Val Thr Val Ser Arg Asp Leu Phe Gly Thr Gly Lys Leu

                      240
GTG CAG CCC GGG GAC CTC ACC CTT GGC TCA GAG GGT TGT CAG CCC
Val Gln Pro Gly Asp Leu Thr Leu Gly Ser Glu Gly Cys Gln Pro

                                          300
CGG GTG TCC TTG GAT ACC GAC GTG GTC AGG TTC AAC GCC CAG TTG
Arg Val Ser Val Asp Thr Asp Val Val Arg Phe Asn Ala Gln Leu

                      330
CAC GAG TGC AGC AGC AGG GTG CAG ATG ACG AAA GAT GCC CTG GTG
His Glu Cys Ser Ser Arg Val Gln Met Thr Lys Asp Ala Leu Val

                                          390
TAC AGC ACC TTC CTA CTC CAC GAC CCT CGC CCT GTG AGT GGC CTG
Tyr Ser Thr Phe Leu Leu His Asp Pro Arg Pro Val Ser Gly Leu

                      420
TCC ATC CTC AGG ACT AAC CGT GTG GAG GTA CCC ATT GAG TGC CGA
Ser Ile Leu Arg Thr Asn Arg Val Glu Val Pro Ile Glu Cys Arg

                                          480
TAC CCC AGG CAG GGC AAT GTG AGC AGC CAC CCT ATC CAG CCC ACC
Tyr Pro Arg Gln Gly Asn Val Ser Ser His Pro Ile Gln Pro Thr

                      510
TGG GTT CCC TTC AGA GCC ACT GTG TCC TCA GAG GAG AAA CTG GCT
Trp Val Pro Phe Arg Ala Thr Val Ser Ser Glu Glu Lys Leu Ala

                                          570
TTC TCT CTT CGC CTG ATG GAG GAG AAC TGG AAT ACT GAG AAA TCG
Phe Ser Leu Arg Leu Met Glu Glu Asn Trp Asn Thr Glu Lys Ser

                      600
GCT CCC ACC TTC CAC CTG GGA GAG GTA GCC CAC CTC CAG GCA GAA
Ala Pro Thr Phe His Leu Gly Glu Val Ala His Leu Gln Ala Glu

                                          660
GTC CAG ACT GGA AGC CAC CTG CCG CTG CAG CTG TTT GTG GAC CAC
Val Gln Thr Gly Ser His Leu Pro Leu Gln Leu Phe Val Asp His
```

690
TGC GTG GCC ACG CCT TCA CCT TTG CCA GAC CCG AAC TCC TCC CCC
Cys Val Ala Thr Pro Ser Pro Leu Pro Asp Pro Asn Ser Ser Pro

750
TAT CAC TTC ATC GTG GAC TTC CAC GGT TGC CTT GTG GAT GGT CTA
Tyr His Phe Ile Val Asp Phe His Gly Cys Leu Val Asp Gly Leu

780
TCT GAG AGC TTT TCG GCA TTT CAA GTC CCC AGA CCC CGG CCA GAG
Ser Glu Ser Phe Ser Ala Phe Gln Val Pro Arg Pro Arg Pro Glu

840
ACT CTC CAG TTC ACG GTG GAT GTA TTC CAT TTT GCC AAC AGC TCC
Thr Leu Gln Phe Thr Val Asp Val Phe His Phe Ala Asn Ser Ser

870
AGA AAT ACG CTC TAC ATC ACC TGC CAT CTC AAA GTC GCG CCA GCT
Arg Asn Thr Leu Tyr Ile Thr Cys His Leu Lys Val Ala Pro Ala

930
AAC CAG ATC CCC GAT AAG CTC AAC AAA GCC TGT TCG TTC AAC AAG
Asn Gln Ile Pro Asp Lys Leu Asn Lys Ala Cys Ser Phe Asn Lys

960
ACT TCC CAG AGT TGG TTG CCA GTA GAG GGT GAT GCT GAC ATC TGT
Thr Ser Gln Ser Trp Leu Pro Val Glu Gly Asp Ala Asp Ile Cys

1020
GAT TGC TGC AGC CAT GGC AAC TGT AGT AAT TCA AGC TCT TCA CAG
Asp Cys Cys Ser His Gly Asn Cys Ser Asn Ser Ser Ser Ser Gln

1050
TTC CAG ATC CAT GGA CCC CGC CAG TGG TCC AAG CTA GTT TCT CGA
Phe Gln Ile His Gly Pro Arg Gln Trp Ser Lys Leu Val Ser Arg

1110
AAC CGC AGG CAC GTG ACC GAT GAA GCT GAT GTC ACT GTA GGG CCC
Asn Arg Arg His Val Thr Asp Glu Ala Asp Val Thr Val Gly Pro

1140
CTG ATA TTC CTT GGA AAG GCC AAC GAC CAG ACT GTG GAA GGC TGG
Leu Ile Phe Leu Gly Lys Ala Asn Asp Gln Thr Val Glu Gly Trp

1200
ACT GCT TCT GCT CAA ACC TCT GTG GCT CTT GGG TTA GGC CTG GCC
Thr Ala Ser Ala Gln Thr Ser Val Ala Leu Gly Leu Gly Leu Ala

1230
ACA GTG GCA TTC CTG ACC CTG GCA GCT ATA GTC CTT GCT GTC ACC
Thr Val Ala Phe Leu Thr Leu Ala Ala Ile Val Leu Ala Val Thr

1290
AGG AAG TGT CAC TCC TCT TCC TAC CTT GTA TCC CTT CCG CAA TAA
Arg Lys Cys His Ser Ser Ser Tyr Leu Val Ser Leu Pro Gln

AAG AAG AAA CTC A 3'

FIG. 1-2

FIG. 2A

```
1     M E L S Y R L F I C L L L W G S T E L C
      |     | |   | | . | | | |   |     | | |
1     M A S S Y F L F L C L L L C G G P E L C

21    Y P Q P L W L L Q G G A S H P E T S V Q
          |   | | | |   | |       |     |
21    N S Q T L W L L P G G T P T P V G S S S

41    P V L V E C Q E A T L M V M V S K D L F
      | |   | | |   | |   | . |   | | : | | |
41    P V K V E C L E A E L V V T V S R D L F

61    G T G K L I R A A D L T L G P E A C E P
      | | | | | :       | | | |   |   | . |
61    G T G K L V Q P G D L T L G S E G C Q P

81    L V S M D T E D V V R F E V G L H E C G
        | | . | |   | | | | |       | | | |
81    R V S V D T   D V V R F N A Q L H E C S

101   N S M Q V T D D A L V Y S T F L L H D P
          . | . |   | | | | | | | | | | | | |
100   S R V Q M T K D A L V Y S T F L L H D P

121   R P V G N L S I V R T N R A E I P I E C
      | | |     | | | . | | | |   | : | | | |
120   R P V S G L S I L R T N R V E V P I E C

141   R Y P R Q G N V S S Q A I L P T W L P F
      | | | | | | | | | | :   |   | | | . | |
140   R Y P R Q G N V S S H P I Q P T W V P F

161   R T T V F S E E K L T F S L R L M E E N
      |   | |   | | | | |   | | | | | | | | |
160   R A T V S S E E K L A F S L R L M E E N

181   W N A E K R S P T F H L G D A A H L Q A
      | |   | |     | | | | | | :   | | | | |
180   W N T E K S A P T F H L G E V A H L Q A

201   E I H T G S H V P L R L F V D H C V A T
      | : : | | | | . | |   | | | | | | | | |
200   E V Q T G S H L P L Q L F V D H C V A T

221       P T P D Q N A S P Y H T I V D F H G
          |   | |   |   | | | |   | | | | | |
220   P S P L P D P N S S P Y H F I V D F H G

239   C L V D G L T D A S S A F K V P R P G P
      | | | | | |   :     | | |   | | | |   |
240   C L V D G L S E S F S A F Q V P R P R P

259   D T L Q F T V D V F H F A N D S R N M
      : | | | | | | | | | | | | |   | | |
260   E T L Q F T V D V F H F A N S S R N T
```

FIG. 2B

```
1    ATGGAGCTGAGCTATAGGCTCTTCATCTGCCTCCTGCTCTGGGGTAGTACTGAGCTGTGC
30   ATGGCGTCAAGCTATTTCCTCTTCCTTTGTCTCCTGCTGTGTGGAGGCCCCGAGCTGTGC

61   TACCCCCAACCCCTCTGGCTCTTGCAGGGTGGAGCCAGCCATCCTGAGACGTCCGTACAG
90   AATTCCCAGACTCTGTGGCTTTTGCCGGGTGGAACTCCCACCCCAGTGGGGTCCTCATCA

121  CCCGTACTGGTGGAGTGTCAGGAGGCCACTCTGATGGTCATGGTCAGCAAAGACCTTTTT
150  CCTGTGAAGGTGGAGTGTCTGGAAGCTGAACTAGTGGTGACTGTCAGTAGAGACCTTTTT

181  GGCACCGGGAAGCTCATCAGGGCTGCTGACCTCACCTTGGGCCCAGAGGCCTGTGAGCCT
210  GGCACGGGGAAGCTGGTGCAGCCCGGGGACCTCACCCTTGGCTCAGAGGGTTGTCAGCCC

241  CTGGTCTCCATGGACACAGAAGATGTGGTCAGGTTTGAGGTTGGACTCCACGAGTGTGGC
270  CGGGTGTCCGTGGATAC     CGACGTGGTCAGGTTCAACGCCCAGTTGCACGAGTGCAGC

301  AACAGCATGCAGGTAACTGACGATGCCCTGGTGTACAGCACCTTCCTGCTCCATGACCCC
327  AGCAGGGTGCAGATGACGAAAGATGCCCTGGTGTACAGCACCTTCCTACTCCACGACCCT

361  CGCCCCGTGGGAAACCTGTCCATCGTGAGGACTAACCGCGCAGAGATTCCCATCGAGTGC
387  CGCCCTGTGAGTGGCCTGTCCATCCTCAGGACTAACCGTGTGGAGGTACCCATTGAGTGC

421  CGCTACCCCAGGCAGGGCAATGTGAGCAGCCAGGCCATCCTGCCCACCTGGTTGCCCTTC
447  CGATACCCCAGGCAGGGCAATGTGAGCAGCCACCCTATCCAGCCCACCTGGGTTCCCTTC

481  AGGACCACGGTGTTCTCAGAGGAGAAGCTGAGTTTCTCTCTGCGTCTGATGGAGGAGAAC
507  AGAGCCACTGTGTCCTCAGAGGAGAAACTGGCTTTCTCTCTTCGCCTGATGGAGGAGAAC

541  TGGAACGCTGAGAAGAGGTCCCCCACCTTCCACCTGGGAGATGCAGCCCACCTCCAGGCA
567  TGGAATACTGAGAAATCGGCTCCCACCTTCCACCTGGGAGAGGTAGCCCACCTCCAGGCA

601  GAAATCCACACTGGCAGCCACGTGCCACTGCGGTTGTTTGTGGACCACTGCGTGGCCACA
627  GAAGTCCAGACTGGAAGCCACCTGCCGCTGCAGCTGTTTGTGGACCACTGCGTGGCCACG

661  CCGACA          CCAGACCAGAATGCCTCCCCTTATCACACCATCGTGGACTTCCATGGC
687  CCTTCACGTTTGCCAGACCCGAACTCCTCCCCCTATCACTTCATCGTGGACTTCCACGGT

715  TGTCTTGTCGACGGTCTCACTGATGCCTCTTCTGCATTCAAAGTTCCTCGACCCGGGCCA
747  TGCCTTGTGGATGGTCTATCTGAGAGCTTTTCGGCATTCAAGTCCCCAGACCCCGGCCA

775  GATACACTCCAGTTCACAGTGGATGTCTTCCACTTTGCTAATGACTCCAGAAACATG
807  GAGACTCTCCAGTTCACGGTGGATGTATTCCATTTTGCCAACAGCTCCAGAAATACG
```

FIG. 3-1

```
                                              30
CAC CTC GGC GCT TTG GTG GTA CCT TCC AAC ATG GCG AGG TGG CAG
                                        Met Ala Arg Trp Gln

                    60                                   90
AGG AAA GCA TCT GTA AGC TCT CCG TGC GGC AGG AGC ATC TAC AGG
Arg Lys Ala Ser Val Ser Ser Pro Cys Gly Arg Ser Ile Tyr Arg

                                     120
TTT CTT TCC CTC TTA TTC ACC CTT GTG ACT TCA GTG AAC TCA GTA
Phe Leu Ser Leu Leu Phe Thr Leu Val Thr Ser Val Asn Ser Val

                    150                                 180
AGC CTT CCT CAG TCC GAG AAT CCT GCC TTC CCA GGC ACT CTC ATT
Ser Leu Pro Gln Ser Glu Asn Pro Ala Phe Pro Gly Thr Leu Ile

                                     210
TGT GAC AAA GAC GAA GTG AGA ATT GAA TTT TCA AGC AGA TTT GAC
Cys Asp Lys Asp Glu Val Arg Ile Glu Phe Ser Ser Arg Phe Asp

                    240                                 270
ATG GAA AAA TGG AAT CCT TCT GTG GTG GAT ACC CTT GGT AGT GAA
Met Glu Lys Trp Asn Pro Ser Val Val Asp Thr Leu Gly Ser Glu

                                     300
ATT TTG AAC TGC ACT TAT GCT CTG GAC TTG GAA AGG TTC GTC CTG
Ile Leu Asn Cys Thr Tyr Ala Leu Asp Leu Glu Arg Phe Val Leu

                    330                                 360
AAG TTC CCT TAC GAG ACC TGC ACT ATA AAA GTG GTT GGT GGA TAC
Lys Phe Pro Tyr Glu Thr Cys Thr Ile Lys Val Val Gly Gly Tyr

                                     390
CAG GTG AAC ATC AGA GTG GGG GAC ACC ACC ACT GAT GTG AGA TAT
Gln Val Asn Ile Arg Val Gly Asp Thr Thr Thr Asp Val Arg Tyr

                    420                                 450
AAA GAT GAC ATG TAT CAT TTC TTC TGT CCA GCT ATT CAA GCA GAG
Lys Asp Asp Met Tyr His Phe Phe Cys Pro Ala Ile Gln Ala Glu

                                     480
ACC CAT GAG ATT TCA GAA ATT GTT GTC TGC AGG AGA GAT CTA ATA
Thr His Glu Ile Ser Glu Ile Val Val Cys Arg Arg Asp Leu Ile

                    510                                 540
TCT TTT TCT TTC CCA CAA CTT TTC TCT AGG CTT GCT GAT GAA AAC
Ser Phe Ser Phe Pro Gln Leu Phe Ser Arg Leu Ala Asp Glu Asn

                                     570
CAG AAT GTA TCT GAG ATG GGA TGG ATT GTT AAG ATT GGC AAT GGT
Gln Asn Val Ser Glu Met Gly Trp Ile Val Lys Ile Gly Asn Gly

                    600                                 630
ACA AGA GCC CAC ATT CTG CCC TTG AAG GAT GCC ATA GTA CAA GGA
Thr Arg Ala His Ile Leu Pro Leu Lys Asp Ala Ile Val Gln Gly

                                     660
TTT AAT CTT CTG ATT GAC AGC CAG AAA GTG ACT CTC CAC GTG CCA
Phe Asn Leu Leu Ile Asp Ser Gln Lys Val Thr Leu His Val Pro
```

FIG. 3-2

690 720
GCC AAT GCT ACT GGA ATA GTT CAC TAT GTG CAA GAG AGC AGC TAT
Ala Asn Ala Thr Gly Ile Val His Tyr Val Gln Glu Ser Ser Tyr

750
CTC TAT ACT GTG CAG CTG GAG CTC TTG TTC TCA ACC ACT GGG CAG
Leu Tyr Thr Val Gln Leu Glu Leu Leu Phe Ser Thr Thr Gly Gln

780 810
AAG ATC GTC TTC TCA TCA CAC GCT ATC TGC GCA CCA GAT CTT TCT
Lys Ile Val Phe Ser Ser His Ala Ile Cys Ala Pro Asp Leu Ser

840
GTG GCT TGT AAT GCT ACA CAC ATG ACT CTC ACT ATA CCA GAA TTT
Val Ala Cys Asn Ala Thr His Met Thr Leu Thr Ile Pro Glu Phe

870 900
CCT GGG AAG CTA GAG TCT GTG GAC TTT GGA CAA TGG AGC ATC CCT
Pro Gly Lys Leu Glu Ser Val Asp Phe Gly Gln Trp Ser Ile Pro

930
GAG GAC CAA TGG CAT GCC AAT GGA ATT GAC AAA GAA GCA ACA AAT
Glu Asp Gln Trp His Ala Asn Gly Ile Asp Lys Glu Ala Thr Asn

960 990
GGC TTG AGA TTG AAT TTC AGA AAA TCT CTC CTG AAA ACT AAA CCC
Gly Leu Arg Leu Asn Phe Arg Lys Ser Leu Leu Lys Thr Lys Pro

1020
TCT GAA AAA TGT CCA TTC TAC CAG TTC TAC CTC TCT TCA CTC AAG
Ser Glu Lys Cys Pro Phe Tyr Gln Phe Tyr Leu Ser Ser Leu Lys

1050 1080
CTG ACC TTC TAC TTC CAA GGG AAC ATG CTA TCC ACA GTG ATA GAT
Leu Thr Phe Tyr Phe Gln Gly Asn Met Leu Ser Thr Val Ile Asp

1110
CCT GAG TGC CAC TGT GAG TCA CCA GTC TCT ATA GAT GAA CTG TGT
Pro Glu Cys His Cys Glu Ser Pro Val Ser Ile Asp Glu Leu Cys

1140 1170
GCA CAG GAT GGG TTT ATG GAC TTT GAG GTC TAC AGC CAC CAA ACA
Ala Gln Asp Gly Phe Met Asp Phe Glu Val Tyr Ser His Gln Thr

1200
AAA CCC GCA CTG AAC CTG GAC ACC CTC CTG GTG GGA AAT TCC TCT
Lys Pro Ala Leu Asn Leu Asp Thr Leu Leu Val Gly Asn Ser Ser

1230 1260
TGC CAG CCT ATT TTC AAG GTG CAG TCT GTG GGG CTT GCA AGG TTT
Cys Gln Pro Ile Phe Lys Val Gln Ser Val Gly Leu Ala Arg Phe

1290
CAC ATA CCT CTG AAT GGA TGT GGA ACA AGG CAG AAA TTT GAA GGT
His Ile Pro Leu Asn Gly Cys Gly Thr Arg Gln Lys Phe Glu Gly

1320 1350
GAT AAA GTC ATC TAT GAG AAT GAA ATA CAT GCT CTC TGG GAA AAC
Asp Lys Val Ile Tyr Glu Asn Glu Ile His Ala Leu Trp Glu Asn

FIG. 3-3

```
                                        1380
CCA CCC TCC AAC ATT GTA TTC AGA AAC AGC GAG TTC AGG ATG ACA
Pro Pro Ser Asn Ile Val Phe Arg Asn Ser Glu Phe Arg Met Thr

                    1410                                1440
GTA AGA TGC TAT TAC ATC AGA GAC AGT ATG CTA CTA AAT GCC CAT
Val Arg Cys Tyr Tyr Ile Arg Asp Ser Met Leu Leu Asn Ala His

                                        1470
GTC AAA GGA CAT CCT TCT CCA GAG GCC TTT GTA AAG CCA GGC CCA
Val Lys Gly His Pro Ser Pro Glu Ala Phe Val Lys Pro Gly Pro

                    1500                                1530
CTG GTG TTG GTC CTA CAA ACA TAC CCA GAC CAA TCC TAC CAA CGG
Leu Val Leu Val Leu Gln Thr Tyr Pro Asp Gln Ser Tyr Gln Arg

                                        1560
CCT TAC AGG AAG GAT GAG TAC CCT CTA GTG AGG TAC CTC CGC CAG
Pro Tyr Arg Lys Asp Glu Tyr Pro Leu Val Arg Tyr Leu Arg Gln

                    1590                                1620
CCA ATC TAC ATG GAA GTG AAG GTC TTG AGC AGG AAC GAT CCC AAC
Pro Ile Tyr Met Glu Val Lys Val Leu Ser Arg Asn Asp Pro Asn

                                        1650
ATC AAG CTG GTC TTA GAT GAC TGC TGG GCA ACT TCT TCT GAG GAC
Ile Lys Leu Val Leu Asp Asp Cys Trp Ala Thr Ser Ser Glu Asp

                    1680                                1710
CCG GCC TCT GCG CCT CAG TGG CAG ATT GTC ATG GAT GGC TGT GAA
Pro Ala Ser Ala Pro Gln Trp Gln Ile Val Met Asp Gly Cys Glu

                                        1740
TAT GAA CTG GAC AAC TAC CGC ACT ACT TTC CAC CCA GCT GGC TCC
Tyr Glu Leu Asp Asn Tyr Arg Thr Thr Phe His Pro Ala Gly Ser

                    1770                                1800
TCT GCA GCC CAT TCC GGT CAC TAC CAG AGG TTT GAT GTG AAG ACT
Ser Ala Ala His Ser Gly His Tyr Gln Arg Phe Asp Val Lys Thr

                                        1830
TTT GCC TTT GTA TCA GAG GCA CGG GGG CTC TCC AGC CTG ATC TAC
Phe Ala Phe Val Ser Glu Ala Arg Gly Leu Ser Ser Leu Ile Tyr

                    1860                                1890
TTC CAC TGC AGT GCC TTG ATC TGT AAC CAA GTC TCT CTT GAC TCC
Phe His Cys Ser Ala Leu Ile Cys Asn Gln Val Ser Leu Asp Ser

                                        1920
CCT CTG TGC TCT GTG ACT TGC CCT GCA TCA CTG AGG AGC AAA CGA
Pro Leu Cys Ser Val Thr Cys Pro Ala Ser Leu Arg Ser Lys Arg

                    1950                                1980
GAG GCC AAC AAA GAA GAC ACA ATG ACG GTT AGC CTT CCA GGA CCT
Glu Ala Asn Lys Glu Asp Thr Met Thr Val Ser Leu Pro Gly Pro

                                        2010
ATT CTC TTG CTG TCA GAT GTC TCT TCA TCC AAA GGT GTT GAC CCC
Ile Leu Leu Leu Ser Asp Val Ser Ser Ser Lys Gly Val Asp Pro
```

FIG. 3-4

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                 2040                                            2070
AGC AGC TCT GAG ATT ACC AAG GAT ATT ATT GCC AAG GAT ATT GCT
Ser Ser Ser Glu Ile Thr Lys Asp Ile Ile Ala Lys Asp Ile Ala

                                    2110
TCT AAA ACA CTG GGT GCT GTG GCT GCA CTA GTG GGC TCA GCT GTC
Ser Lys Thr Leu Gly Ala Val Ala Ala Leu Val Gly Ser Ala Val

                 2130                                        2160
ATT CTA GGC TTC ATC TGT TAC CTG TAT AAG AAA AGA ACT ATA AGG
Ile Leu Gly Phe Ile Cys Tyr Leu Tyr Lys Lys Arg Thr Ile Arg

                                    2190
TTC AAT CAC TGA TTG GAC TTG CAA ATA AAG AGA CTG CAG TC
Phe Asn His
```

FIG. 4A

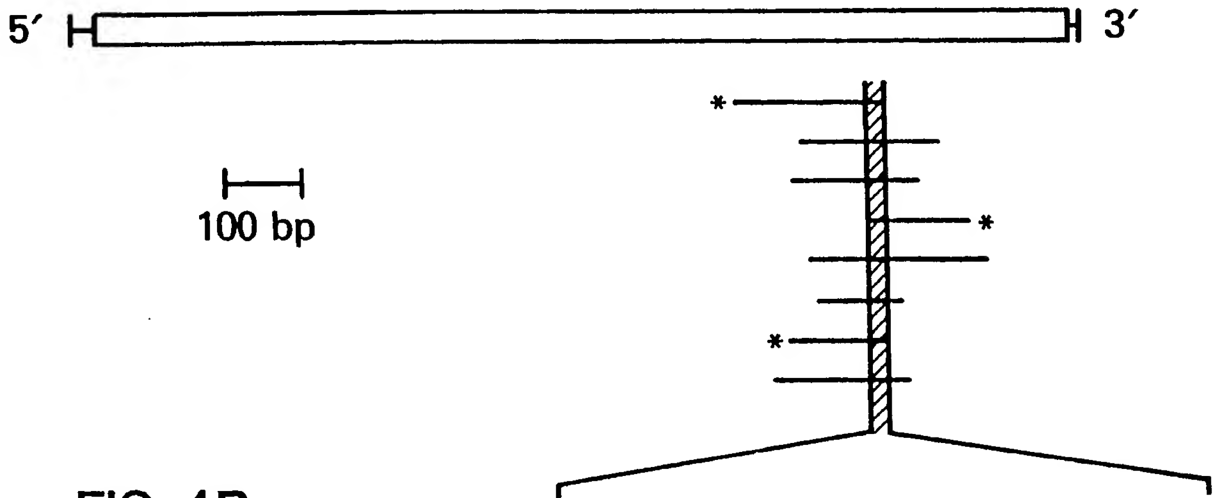
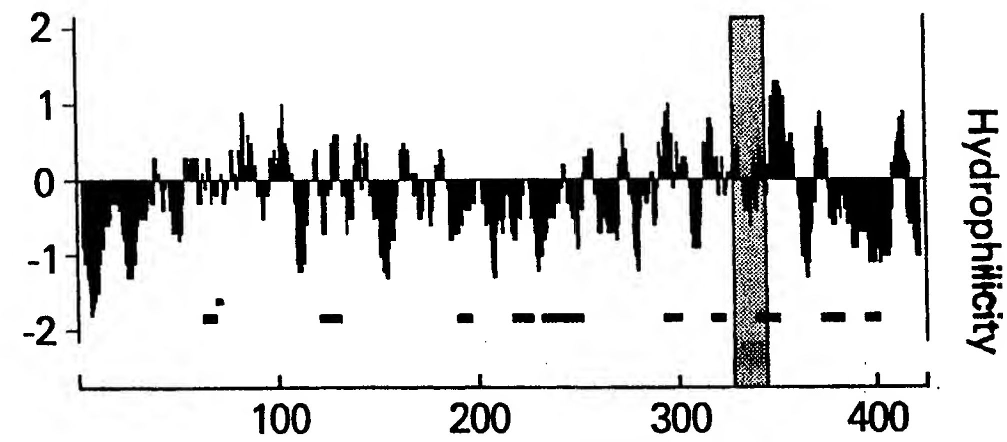


FIG. 4B

AG TTC CAG ATC CAT GGA CCC CGC C

NH_2-cys ser asn ser ser ser ser gln PHE GLN ILE HIS GLY PRO ARG gln-COOH

FIG.4C



INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/03075

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 39/385; C07K 13/00, 15/28; C12N 15/12, 1/21
U.S.Cl.: 536/27; 435/240.1,320;530/387

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	424/88; 536/27; 435/240.1,320; 530/387

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[5]

Automated Patent Search, Swiss-prot and PIR Protein Databases.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X	US, A, 4,795,634 (GRIMES et al.) 03 January 1989, see claims 1-10.	1-13, 18 25, 26
X	US, A, 3,992,520 (GWATKIN) 16 November 1976, see claims 1-7.	1-15, 18 25, 26
X,P	Science, "Vaccination with a Synthetic Zona Pellucida Peptide Produces Long-Term Contraception in Female Mice" vol. 246 pages 935-938. Millar et al., 17 November 1989. See entire article.	1-26
Y / X	Proc. Natl. Acad. Sci. USA, "Oocyte-specific gene expression: Molecular characterization of a cDNA coding for ZP-3, the sperm receptor of the mouse zona pellucida" vol. 83, pages 4341-4345. Rimgiette et al., June 1986. See abstract.	1-13, 18 / 19-26
Y / X	Proc. Natl. Acad. Sci. USA, "Primary structure of the mouse sperm receptor polypeptide determined by genomic cloning". vol. 85, pages 6409-6413, Kinloch et al., September 1988. See abstract.	1-13,18

* Special categories of cited documents:[15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
22 August 1990	01 OCT 1990
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	Nina Ossanna

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y / X	Proc. Natl. Acad. Sci. USA, "Efficient mapping of protein antigenic determinants" vol. 83 pages 7013-7017. Mehra et al., September 1986. See entire article for methodology.	1-13, 18 / 19-26

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter[1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out[1], specifically:

3. ☐ Claim numbers___________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.